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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(51) International Patent Classification <sup>5</sup>:</b><br>A01N 63/00, 43/04, A61K 37/00, 31/70,<br>C07H 17/00, 19/00, 21/00, C07K 3/00,<br>13/00, 15/00, 17/00, C12N 1/20, 5/00,<br>15/00, C12Q 1/00, 1/02, G01N 33/53  | <b>A1</b> | <b>(11) International Publication Number:</b> WO 94/21126<br><b>(43) International Publication Date:</b> 29 September 1994 (29.09.94)  |
| <b>(21) International Application Number:</b> PCT/US94/02487<br><b>(22) International Filing Date:</b> 9 March 1994 (09.03.94)<br><b>(30) Priority Data:</b> 5620886<br>08/032,978 18 March 1993 (18.03.93) US<br><b>(71) Applicant:</b> LUDWIG INSTITUTE FOR CANCER RE-<br>SEARCH [US/US]; 1345 Avenue of the Americas, New<br>York, NY 10105 (US).<br><b>(72) Inventors:</b> BRICHARD, Vincent; Avenue Hippocrate 74,<br>UCL 7459, B-1200 Brussels (BE). VAN PEL, Aline;<br>Avenue hippocrate 74, UCL 7459, B-1200 Brussels (BE).<br>TRAVERSARI, Catia; I-20099 Sesto S. Giovanni (IT).<br>WOLFEL, Thomas; Langenbeckstrasse 1, D-6500 Mainz<br>(DE). BOON-FALLEUR, Thierry; Avenue Hippocrate 74,<br>UCL 7459, B-1200 Brussels (BE). DE PLAEN, Etienne;<br>Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE).<br><b>(74) Agent:</b> HANSON, Norman, D.; Felfe & Lynch, 805 Third<br>Avenue, New York, NY 10022 (US). |           | <b>(81) Designated States:</b> AU, CA, FI, JP, NO, NZ, European patent<br>(AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC,<br>NL, PT, SE).<br><br><b>Published</b><br><i>With international search report.</i> |
| <b>(54) Title:</b> NUCLEIC ACID CODING FOR A TUMOR REJECTION ANTIGEN PRECURSOR<br><br><b>(57) Abstract</b><br><br>The invention relates to nucleic acid molecules coding for a tumor rejection antigen precursor. Specifically, the tumor rejection antigen precursor, or "TRAP", is processed into at least one tumor rejection antigen, which is presented by HLA-A2 molecules. Ramifications of the discovery are also set forth.   |           |  |

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NUCLEIC ACID CODING FOR A TUMOR  
REJECTION ANTIGEN PRECURSOR

5      **FIELD OF THE INVENTION**

         This invention relates to a nucleic acid molecule which codes for a tumor rejection antigen precursor. More particularly, the invention concerns a gene, whose tumor rejection antigen precursor is processed, inter alia, into at least one tumor rejection antigen that is presented by HLA-A2 molecules on cell surfaces.

10      **BACKGROUND AND PRIOR ART**

         The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cell and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Recently, much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257: 880 (1992); Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992).

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991), for further information on this family of genes.

In U.S. patent application Serial Number 938,334, the disclosure of which is incorporated by reference, nonapeptides are taught which are presented by the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent Application Serial Number 008,446, filed January 22, 1993 and incorporated by reference, the fact that the MAGE-1 expression product is processed to a second TRA is disclosed. This second TRA is presented by HLA-C10-molecules. The disclosure shows that a given TRAP can yield a plurality of TRAs.

In U.S. Patent Application Serial Number 994,928, filed December 22, 1992, and incorporated by reference herein,

tyrosinase is described as a tumor rejection antigen precursor. This reference discloses that a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield a tumor rejection antigen that is presented by HLA-A2 molecules.

It has now been found that another nucleic acid molecule codes for a tumor rejection antigen precursor which differs from those described previously. The TRAP of the invention is processed to at least one tumor rejection antigen that is presented by HLA-A2 molecules; however sequence analysis indicates that the TRAP of the invention is not, nor is it related to, tyrosinase. Thus the invention relates to a nucleic acid molecule which codes for a tumor rejection antigen precursor, or "TRAP" molecule. This "TRAP" molecule is not tyrosinase. Further, the TRAP of the invention is processed to at least one tumor rejection antigen, or "TRA", which is presented by HLA-A2 molecules. The TRA is not tyrosinase related, and other TRAs derived from the TRAPs of the invention may be presented by other HLA molecules.

The invention and various aspects thereof will be elaborated upon in the disclosure which follows.

#### **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1A presents results of cell lysis experiments using CTL clone I/95 against LB39-MEL, K562, and LB39 blasts.

Figure 1B shows lysis using CTL clone I/95 against SK23-MEL and SK29-MEL.

Figure 2 sets forth results of a TNF release assay using various cell lines with CTL I/95.

Figure 3A shows TNF release induced by different cell lines, including transfectants, when tested with CTL clone I/95.

Figure 3B presents TNF release data using CTL clone IVSB.

Figure 3C shows TNF release using CTL clone 10/196.

Figure 4 presents a panel of tissues, cell lines and tumors tested for expression of the gene AaG1c124 using polymerase chain reaction (PCR) using oligonucleotide probes

derived from the nucleic acid molecule described herein.

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

**Example 1**

5 A melanoma cell line, "LB-39-MEL" was established from melanoma cells taken from patient LB39, using standard methodologies. Once the cell line was established, a sample thereof was irradiated, so as to render it non-proliferative. These irradiated cells were then used to isolate cytolytic T cells ("CTLs") specific thereto.

10 A sample of peripheral blood mononuclear cells ("PBMCs") was taken from patient LB39, and contacted to the irradiated melanoma cells. The mixture was observed for lysis of the melanoma cells, which indicated that CTLs specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

15 The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39:390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown in vitro, and then resuspended at  $10^7$  cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200  $\mu$ Ci/ml of Na( $^{51}$ Cr)O<sub>4</sub>. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100  $\mu$ l aliquots containing  $10^3$  cells, were distributed into 96 well microplates. Samples of PBLs were added in 100  $\mu$ l of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in a 80% of CO<sub>2</sub> atmosphere.

25 Plates were centrifuged again, and 100  $\mu$ l aliquots of supernatant were collected and counted. Percentage of  $^{51}$ Cr release was calculated as follows:

$$\% \text{ } ^{51}\text{Cr release} = \frac{(\text{ER}-\text{SR})}{(\text{MR}-\text{SR})} \times 100$$

35 where ER is observed, experimental  $^{51}$ Cr release, SR is spontaneous release measured by incubating  $10^3$  labeled cells

in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology. The CTL clone LB39-CTL I/95 was thus isolated.

The same method was used to test target K562 cells, as well as autologous, PHA induced T cell blasts. These results, presented in Figure 1A, show that this CTL clone recognizes and lyses the melanoma cell line, but neither of K562 or the T cell blasts. The CTL, LB39-CTL I/95, was then tested against melanoma cell lines SK23-MEL and SK29 MEL, in the same manner described supra. Cells from both of these lines were also lysed. These lines were both isolated from patients who were typed as HLA-A2, as was LB39. This suggested that the CTL clone LB39-CTL I/95 recognized an antigen presented by HLA-A2.

#### Example 2

Further studies were carried out to determine if LB39-CTL I/95 also produced tumor necrosis factor ("TNF") when contacted with target cells. The method used was that described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. Briefly, samples of the CTL line were combined with samples of a target cell of interest, in culture medium. After 24 hours, supernatant from the cultures was removed, and then tested on TNF sensitive WEHI cells. In addition to LB39-MEL and SK23-MEL, described supra, another HLA-A2 line, i.e., SK29-MEL.1, an HLA-A2 loss variant, i.e., SK29-MEL1.22, and a non HLA-A2 line, i.e., MZ2-MEL, which is HLA-A1, were tested.

The results, presented in terms of the percentage of WEHI cells which died upon exposure to the supernatant, are shown in Figure 2. These results show that the HLA-A2 loss variant SK 29-MEL.1.22 is no longer capable of stimulating the CTL clone, thus confirming that the antigen recognized by LB39-CTL-I/95 is presented by HLA-A2.

#### Example 3

The results from Example 2 indicated that SK MEL 29.1 presented the target antigen of interest. As such, it was used as a source of total mRNA to prepare a cDNA library.

5 Total RNA was isolated from the cell line. The mRNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the mRNA was secured, it was transcribed into cDNA, again using standard methodologies. The cDNA was then ligated to EcoRI adaptors and cloned into the EcoRI site of plasmid pcDNA-I/Amp, in accordance with  
10 manufacturer's instructions. The recombinant plasmids were then electroporated into JM101 *E. coli* (electroporation conditions: 1 pulse at 25  $\mu$ farads, 2500 V).

The transfected bacteria were selected with ampicillin (50  $\mu$ g/ml), and then divided into 800 pools of 100 clones  
15 each. Each pool represented about 50 different cDNAs, as analysis showed that about 50% of plasmids contained an insert. Each pool was amplified to saturation, and plasmid DNA was isolated via alkaline lysis, potassium acetate precipitation without phenol extraction, following Maniatis et  
20 al., in Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, N.Y., 1982).

#### Example 4

Following preparation of the library described in Example 3, the cDNA was transfected into eukaryotic cells. The  
25 transfections, described herein, were carried out in duplicate. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at  
30 37°C, medium was removed and then replaced by 30  $\mu$ l/well of DMEM medium containing 10% Nu serum, 400  $\mu$ g/ml DEAE-dextran, 100  $\mu$ M chloroquine, 100 ng of plasmid pcDNA-I/Amp-A2 and 100 ng of DNA of a pool of the cDNA library described supra. Plasmid pcDNA-I/Amp-A2 contains the HLA-A2 gene from SK29-MEL.  
35 Following four hours of incubation at 37°C, the medium was removed, and replaced by 50  $\mu$ l of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200

μl of DMEM supplement d with 10% f FCS.

Following this change in medium, COS cells were incubated for 48 hours at 37°C. Medium was then discarded, and 1000 cells of CTL I/95 were added, in 100 μl of Iscove medium containing 10% pooled human serum, supplemented with 25 U/ml of IL-2. Supernatant was removed after 24 hours, and TNF content was determined in the assay on WEHI cells, as described by Traversari et al., supra, previously incorporated by reference.

Of the 800 pools tested, 99% stimulated TNF release, to a concentration of from 3-6 pg/ml in the supernatant. Two pools gave yields over 8 pg/ml, with a duplicate well also yielding over 8 pg/ml.

#### Example 5

The two pools showing high yields of TNF in the supernatant were selected for further study. Specifically, the bacteria were cloned, and 800 bacteria were tested from each pool. Plasmid DNA was extracted therefrom, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of LB39-CTL clone I/95. One positive clone was found, referred to as AaG1cl24. Convincing evidence that the transfected cells were recognized by CTLs was obtained by carrying out a comparative test of COS cells transfected with cDNA from the positive clone and the HLA-A2 gene, COS cells transfected only with HLA-A2, and line SK29-MEL. TNF release in CTL supernatant was measured by testing it on WEHI cells, as referred to supra. The optical density of the surviving WEHI cells was measured using MTT. Figure 3A shows the results obtained with CTL clone I/95.

Further tests showed that the peptide presented by HLA-A2 in the transfected cells was different from that observed previously, i.e., a tyrosinase derived peptide. CTL clone IVSB is specific to complexes of tyrosinase derived peptide and HLA-A2. When this CTL clone was contacted to cells transfected with AaG1cl24 and HLA-A2, TNF release was minimal, as shown in Figure 3B.

Example 6

The cDNA from the positive clone was removed, and sequenced following art known techniques. A sequence search revealed that the plasmid insert showed no homology to known genes or proteins. SEQUENCE ID NO: 1 presents cDNA nucleotide information, showing a large, open reading frame from positions 75 to 431, corresponding to a protein product of 119 amino acids. Sequence ID NO: 2 sets forth the extended sequence of which SEQ ID NO: 1 is a part.

Example 7

In the same manner that CTL clone LB39-CTL I/95 was isolated, a sample of PBMCs and a melanoma cell line developed from patient SK29(AV) were used to isolate CTL clone SK29-CTL 10/196. This new cell line was tested in the same manner as is set forth in Example 5. The results of the assays, depicted in Figure 3C, show that the tumor rejection antigen coded for by AaG1cl24 (referred to as antigen "LB39-Aa" hereafter), is also recognized by this CTL clone. These experiments indicate that other patients can, and in fact do, generate CTLs specific for this antigen.

Oligonucleotide probes were derived from the described sequences, and were used in standard polymerase chain reaction methodologies to determine expression of the gene in normal tissues, tumors, and tumor cell lines. These results are presented in figure 4, and show that among normal tissues tested, only melanocytes expressed the gene. Note the expression in all tumor samples and/or melanoma cell lines tested.

The foregoing experiments describe a newly isolated nucleic acid sequence coding for a tumor rejection antigen precursor, a "TRAP" molecule. The molecule is processed intracellularly in a manner which leads to production of at least one tumor rejection antigen, or "TRA", which is presented by HLA-A2 molecules. While it has been observed previously that HLA-A2 molecules present peptides derived from tyrosinase, the nucleic acid sequences of the invention do not code for tyrosinase, and the TRAs are not tyrosinase derived.

The invention thus involves an isolated nucleic acid molecule which codes for a tumor rejection antigen precursor, or "TRAP", with the proviso that the TRAP is not tyrosinase. The TRAP coded for is one which is processed to at least one tumor rejection antigen, or TRA, which is presented by HLA-A2 molecules on cell surfaces. The nucleic acid molecules of the invention may be, e.g., genomic DNA, ("gDNA"), complementary DNA ("cDNA"), or a form of RNA. The invention also involves isolated nucleic acid molecules which are complementary to the molecules described above. An especially preferred form of the invention is a molecule which contains the sequence set forth in SEQ. ID NO: 1.

Also encompassed by the invention are vectors which contain the nucleic acid molecules of the invention, operably linked to a promoter. The vectors may also include a molecule coding for HLA-A2. As these two molecules, i.e., HLA-A2 and the TRAP are necessary to generate a cytolytic T cell response, the invention also encompasses expression systems where nucleic acid molecules coding for TRAP and for HLA-A2 are presented as separate portions in, e.g., a kit. The invention also encompasses cell lines transfected by the vectors described herein, be these prokaryotic cells, such as *E. coli*, or eukaryotic cells, such as Chinese hamster ovary ("CHO") or COS cells.

As indicated, the complexes of TRA and HLA-A2 provoke a cytolytic T cell response, and as such isolated complexes of the tumor rejection antigen and an HLA-A2 molecule are also encompassed by the invention, as are isolated tumor rejection antigen precursors coded for by the previously described nucleic acid sequences.

The invention as described herein has a number of uses, some of which are described herein. First, the identification of a tumor rejection antigen which is specifically presented by HLA-A2 molecules, as well as a nucleic acid molecule coding for its parallel tumor rejection antigen precursor permits the artisan to diagnose a disorder characterized by expression of the TRAP. These methods involve determining expression of the

TRAP gene, and/or TRAs deriv d therefrom, such as TRA presented by HLA-A2. Other TRAs may als be derived from the TRAPs of the invention and presented by different HLA molecules. In the former situation, such determinations can  
5 be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labelled hybridization probes. In the latter situation, assaying with binding partners for complexes of TRA and HLA, such as antibodies, is especially preferred.

10 The isolation of the TRAP gene also makes it possible to isolate the TRAP molecule itself, especially TRAP molecules containing the amino acid sequence of SEQ ID NO: 1. These isolated molecules when presented as the TRA, or as complexes of TRA and HLA, such as HLA-A2, may be combined with materials  
15 such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule. In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-proliferative cancer cells, non-proliferative transfectants,  
20 etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to prove a CTL response, or be cells which express both molecules without transfection. Further, the TRAP molecule, its associated TRAs, as well as  
25 complexes of TRA and HLA, may be used to produce antibodies, using standard techniques well known to the art.

30 When "disorder" is used herein, it refers to any pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer melanoma in particular.

Therapeutic approaches based upon the disclosure are premised on a response by a subject's immune system, leading to lysis of TRA presenting cells, such as HLA-A2 cells. One such approach is the administration of CTLs specific to the  
35 complex to a subject with abnormal cells of the phenotype at issu . it is within the skill of the artisan to dev lop such CTLs in vitro. Specifically, a sample of cells, such as blood

cells, are contacted to a cell presenting the complex and capable of providing a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein are widely available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Reddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA containing the indicated sequences. Once isolated, such cells can be used with a sample of a subject's abnormal cells to determine lysis in vitro. If lysis is observed, then the use of specific CTLs in such a therapy may alleviate the condition associated with the abnormal cells. A less involved methodology examines the abnormal cells for HLA phenotyping, using standard assays, and determines expression via amplification using, e.g., PCR.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in

5 this approach may be those that normally express the complex,  
such as irradiated melanoma cells or cells transfected with  
one or both of the genes necessary for presentation of the  
complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114  
10 (January, 1991) exemplifies this approach, showing the use of  
transfected cells expressing HPVE7 peptides in a therapeutic  
regime. Various cell types may be used. Similarly, vectors  
carrying one or both of the genes of interest may be used.  
Viral or bacterial vectors are especially preferred. In these  
15 systems, the gene of interest is carried by, e.g., a Vaccinia  
virus or the bacteria BCG, and the materials de facto "infect"  
host cells. The cells which result present the complex of  
interest, and are recognized by autologous CTLs, which then  
proliferate. A similar effect can be achieved by combining  
20 the tumor rejection antigen or the precursor itself with an  
adjuvant to facilitate incorporation into HLA-A2 presenting  
cells which present the HLA molecule of interest. The TRAP is  
processed to yield the peptide partner of the HLA molecule  
while the TRA is presented without the need for further  
processing.

Other aspects of the invention will be clear to the  
skilled artisan and need not be repeated here.

25 The terms and expressions which have been employed are  
used as terms of description and not of limitation, and there  
is no intention in the use of such terms and expressions of  
excluding any equivalents of the features shown and described  
or portions thereof, it being recognized that various  
modifications are possible within the scope of the invention.

## (1) GENERAL INFORMATION:

5 (i) APPLICANTS: Brichard, Vincent, Van Pel, Aline,  
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10 (ii) TITLE OF INVENTION: ISOLATED NUCLEIC ACID SEQUENCE CODING  
FOR A TUMOR REJECTION ANTIGEN PRECURSOR PROCESSED TO AT LEAST ONE  
TUMOR REJECTION ANTIGEN PRESENTED BY HLA-A2

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:  
25 (A) MEDIUM TYPE: Diskette, 5.25 inch, 360 kb storage  
(B) COMPUTER: IBM PS/2  
(C) OPERATING SYSTEM: PC-DOS  
(D) SOFTWARE: Wordperfect

30 (vi) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/032,978  
(B) FILING DATE: 18-Mar-1993

35 (vii) ATTORNEY/AGENT INFORMATION:  
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45

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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 354 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

|    |   |     |
|----|---|-----|
| 10 | ATG CGA AGA GAA GAT GCT CAC TTC ATC TAT GGT TAC CCC AAG AAG GGG | 48  |
|    | Met Pro Arg Glu Asp Ala His Phe Ile Tyr Gly Tyr Pro Lys Lys Gly |     |
|    | 5 10 15   |     |
| 15 | GAC GGC CAC TCT TAC ACC ACG GCT GAA GAG GCC GCT GGG ATC GGC ATC | 96  |
|    | His Gly His Ser Tyr Thr Thr Ala Glu Glu Ala Ala Gly Ile Gly Ile |     |
|    | 20 25 30  |     |
| 20 | CTG ACA GTG ATC CTG GGA GTC TTA CTG CTC ATC GGC TGT TGG TAT TGT | 144 |
|    | Leu Thr Val Ile Leu Gly Val Leu Leu leu Ile Gly Cys Trp Tyr Cys |     |
|    | 35 40 45  |     |
| 25 | AGA AGA CGA AAT GGA TAC AGA GCC TTG ATG GAT AAA AGT CTT CAT GTT | 192 |
|    | Arg Arg Arg Asn Gly Tyr Arg Ala Leu Met Asp Lys Ser Leu His Val |     |
|    | 50 55 60  |     |
| 30 | GGC ACT CAA TGT GCC TTA ACA AGA AGA TGC CCA CAA GAA GGG TTT GAT | 240 |
|    | Gly Thr Gln Cys Ala Leu Thr Arg Arg Cys Pro Gln Glu Gly Phe Asp |     |
|    | 65 70 75 80   |     |
| 35 | CAT CGG GAC AGC AAA GTG TCT CTT CAA GAG AAA AAC TGT GAA CCT GTG | 288 |
|    | His Arg Asp Ser Lys Val Ser Leu Gln Glu Lys Asn Cys Glu Pro Val |     |
|    | 85 90 95  |     |
| 40 | GTT CCC AAT GCT CCA CCT GCT TAT GAG AAA CTC TCT GCA GAA CAG TCA | 336 |
|    | Val Pro Asn Ala Pro Pro Ala Tyr Glu Lys Leu Ser Ala Glu Gln Ser |     |
|    | 100 105 110   |     |
| 45 | CCA CCA CCT TAT TCA CCT   | 354 |
|    | Pro Pro Pro Tyr Ser Pro   |     |
|    | 115   |     |

15

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 676 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
10      TCTTCATACA CGCGGCCAGC CAGCAGACAG AGGACTCTCA TTAAGGAAGG TGTCTGTGTC 60
      CCTGACCCTA CAAGATGCCA AGAGAAGATG CTCACTTCAT CTATGGTTAC CCCAAGAAGG 120
      GGCACGGCCA CTCCTACACC ACGGCTGAAC AGGCCGCTGG GATCGGCATC CTGACAGTGA 180
      TCCTGGGAGT CTTACTGCTC ATCGGCTGTT GGTATTGTAG AAGACGAAAT GGATACAGAG 240
15      CCTTGATGGA TAAAAGTCTT CATGTTGGCA CTCAATGTGC CTTAACAAGA AGATGCCCAC 300
      AAGAAGGGTT TGATCATCGG GACAGCAAAG TGTCTCTTCA AGAGAAAAAC TGTGAACCTG 360
      TGGTTCCCAA TGCTGCAGGT GCTTATGAGA AACTCTCTGC AGAACAGTCA GGACCACCTT 420
      ATTCACCTTA AGAGCCAGCG AGACACCTGA GACATGGCTG AAATTATTTT TCTCACACTT 480
      TTGCTTGAAT TTAATACAGA CATCTAATGT TCTCCTTTGG AATCCTGTAG GAAAAATGCA 540
20      AGCCATCTCT AATAATAAGT CAGTGTTAAA ATTTTAGTAG GTCCGCTAGC AGTACTAATC 600
      ATGTGAGGAA ATGATGAGAA ATATTAAATT GGGAAAACTC CATCAATAAA TGTGCAAAT 660
      GCATAGTAAA AAAAAA                                     676
```

W claim:

1. Isolated nucleic acid molecule which codes for or is complementary to a nucleic acid molecule which codes for a tumor rejection antigen precursor which is processed to a tumor rejection antigen presented by HLA-A2 molecules.

2. The isolated nucleic acid molecule of claim 1, wherein said nucleic acid molecule codes for said tumor rejection antigen precursor.

3. The isolated nucleic acid molecule of claim 2, wherein said nucleic acid molecule is DNA.

4. The isolated nucleic acid molecule of claim 3, wherein said DNA is cDNA.

5. The isolated nucleic acid molecule of claim 4, comprising the nucleotide sequence of SEQ ID NO: 1.

6. Vector comprising the nucleic acid molecule of claim 1 operably linked to a promoter.

7. The vector of claim 6, comprising the nucleotide sequence of SEQ ID NO: 1.

8. Cell line transfected with the isolated nucleic acid molecule of claim 1.

9. The cell line of claim 8, wherein said nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 1.

10. The cell line of claim 8, transfected with a vector.

11. The cell line of claim 10, wherein said vector comprises the nucleotide sequence of SEQ ID NO: 1.

12. The cell line of claim 8, transfected with a nucleic acid molecule which codes for HLA-A2.

13. The vector of claim 6, further comprising a nucleic acid molecule which codes for HLA-A2.

14. Method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor which is processed by a tumor rejection antigen presented by HLA-A2 molecules, comprising: administering to said subject an amount of cytolytic T cells which are specific for complexes of said tumor rejection antigen and HLA-A2 molecules and which lyse cells presenting said complexes, sufficient to alleviate said disorder.

15. Method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor coded for by a nucleic acid molecule and comprising the nucleotide sequence of SEQ ID NO: 1, comprising administering to said subject an amount of cytolytic T cells specific to complexes of an HLA molecule and a tumor rejection antigen derived from said tumor rejection antigen precursor, sufficient to alleviate said disorder.

16. Method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor which is processed to a tumor rejection antigen presented by HLA-A2 molecules, comprising administering to said subject an amount of an agent which provokes an immune response to complexes of said tumor rejection antigen and HLA-A2 molecules sufficient to provoke said response against cells presenting said complex.

17. Method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor coded for by a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1, comprising administering to said subject an amount of an agent which provokes an immune response to complexes of an HLA molecule and a tumor rejection antigen precursor, sufficient to provoke said immune response against cells presenting said complexes.

18. Method for diagnosing a disorder characterized by expression of a tumor rejection antigen precursor which is processed to a tumor rejection antigen which forms a complex with HLA-A2 molecules, comprising contacting a sample from a subject with an agent specific for said complex and determining interaction between said complex and said agent as a determination of said disorder.

19. Method for diagnosing a disorder characterized by expression of a tumor rejection antigen precursor coded for by a nucleic acid molecule having the sequence set forth in SEQ ID NO: 1, comprising contacting a sample from a subject with an antigen specific for said sequence or an expression product thereof, and determining interaction between said agent and

said sequence or said expression product as a determination of said disorder.

20. Isolated tumor rejection antigen precursor coded for by the nucleic acid molecule of claim 2.

FIG. 1A'

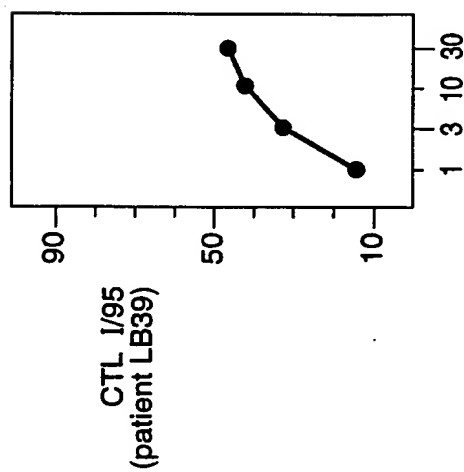


FIG. 1A''

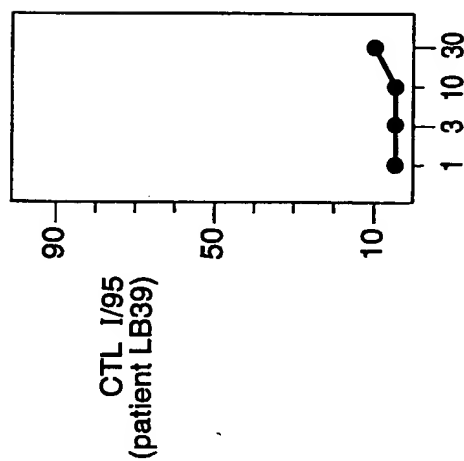
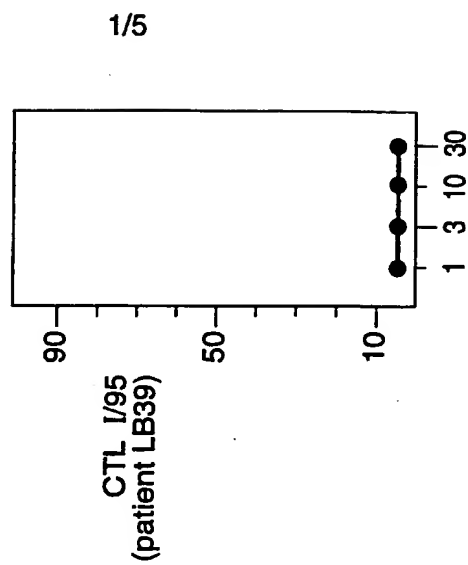


FIG. 1A'''



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FIG. 1B''

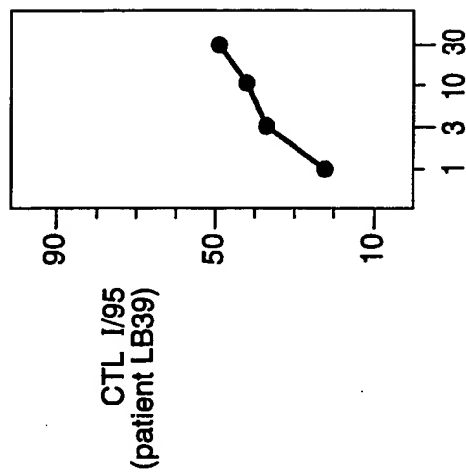
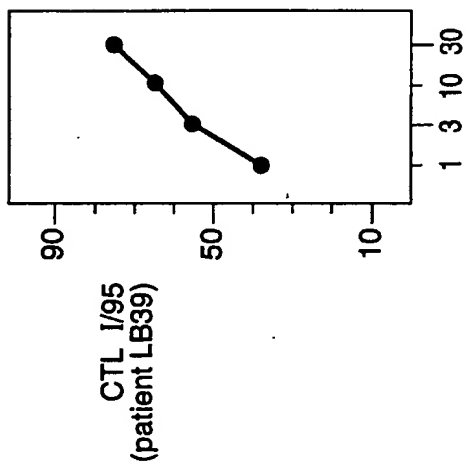
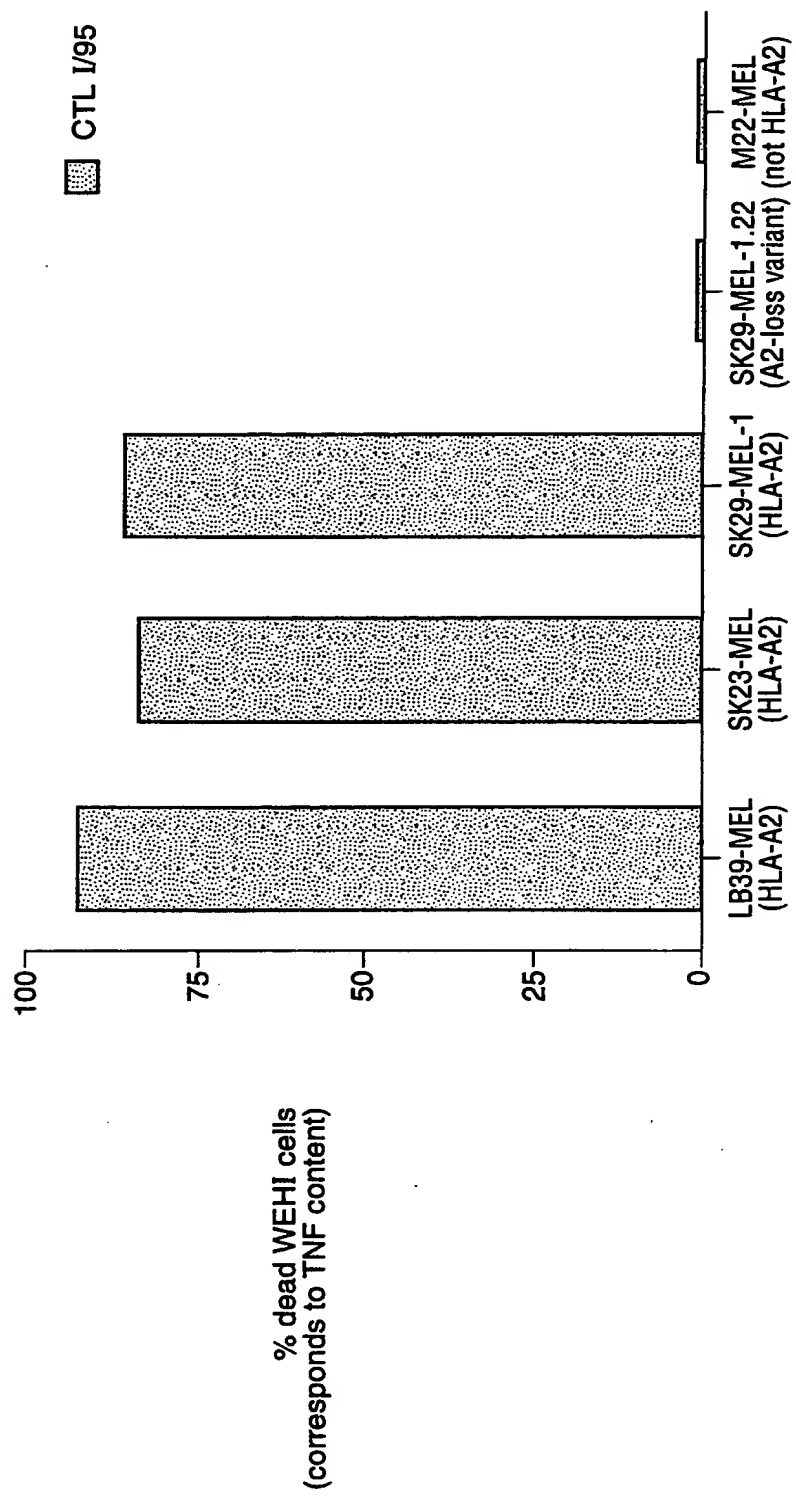


FIG. 1B'



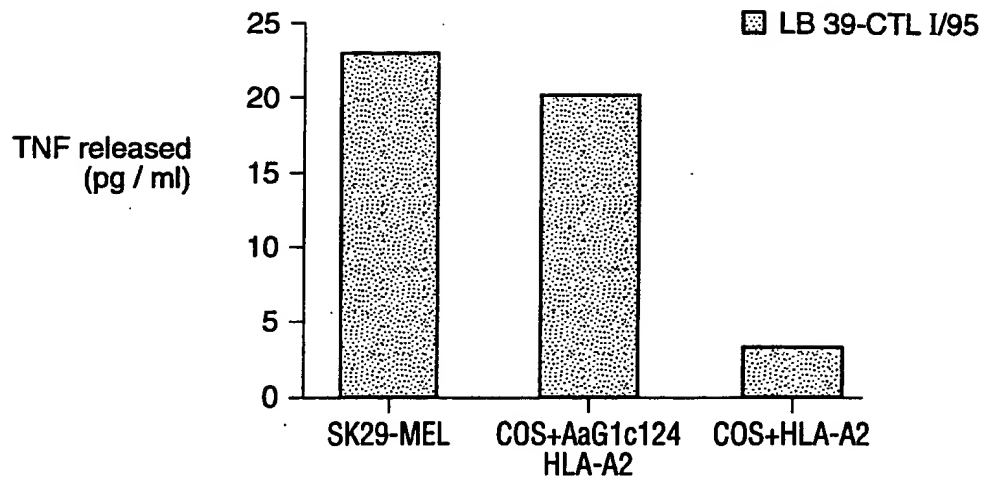
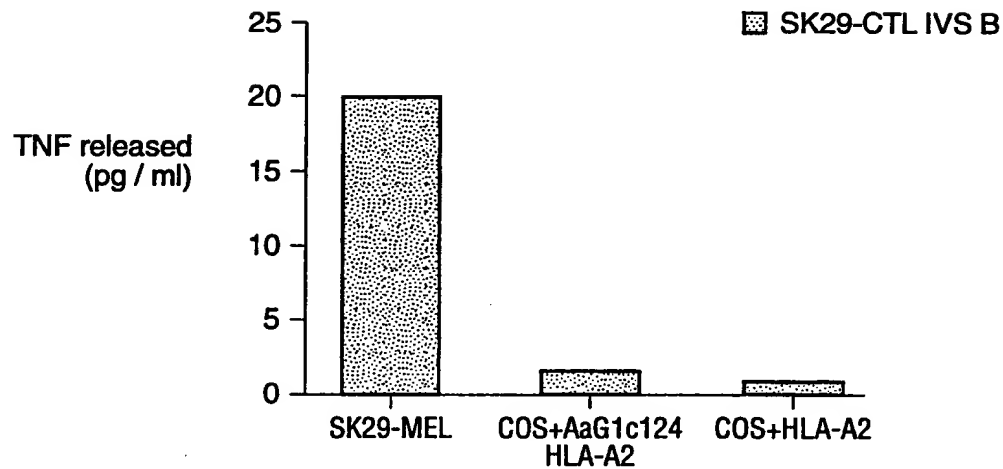
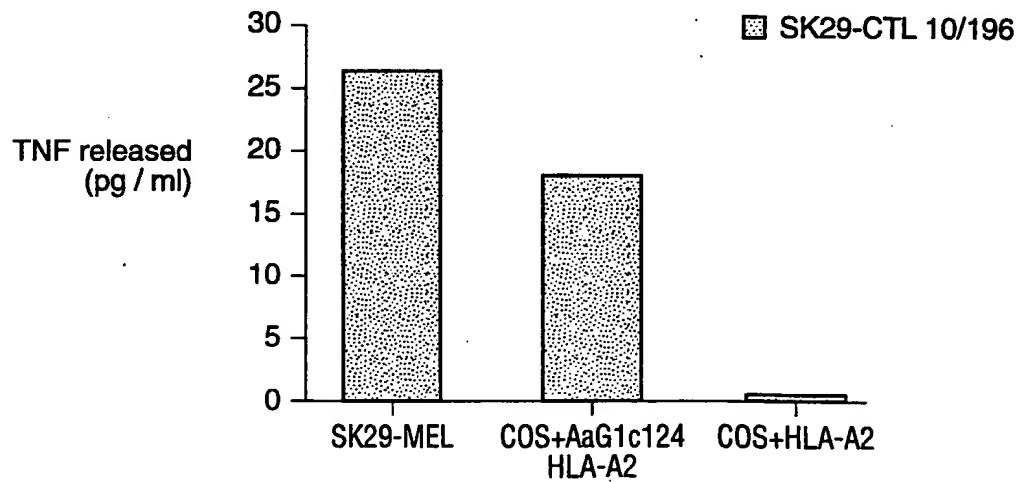
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**FIG. 2**



**FIG. 3A**

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**FIG. 3B****FIG. 3C**

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**FIG. 4****NORMAL TISSUES****As**

|                        |   |
|------------------------|---|
| melanocytes SK         | + |
| heart                  | - |
| liver                  | - |
| kidney BA4             | - |
| prostate Clontech      | - |
| adrenals               | - |
| adrenals               | - |
| adrenals               | - |
| adrenals pool Clontech | - |
| testis Clontech        | - |
| testis LB451           | - |
| brain Clontech         | - |
| fetal brain Clontech   | - |
| lung LB175             | - |
| lung LB195             | - |
| skin LB 177            | - |
| CTL SK29 IVS B         | - |

|   |
|---|
| + |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |
| - |

**MELANOMA****CELL LINES**

|           |
|-----------|
| LB24-MEL  |
| SK23-MEL  |
| LE518-MEL |
| LB38-MEL  |
| MZ13-MEL  |
| LB33-MEL  |
| LB3-MEL   |

|   |
|---|
| + |
| + |
| + |
| + |
| - |
| - |
| + |

**TUMOR SAMPLES**

|           |
|-----------|
| LB239-MEL |
| LB15-MEL  |
| LB492-MEL |
| LB503-MEL |
| LB435-MEL |
| LB224-MEL |
| LG18-MEL  |

|   |
|---|
| + |
| + |
| + |
| + |
| + |
| + |
| + |

**OTHER TUMOR CELL LINES**

|                       |
|-----------------------|
| LB23 sarcoma          |
| LE89.15 kidney tumor  |
| BT20 breast carcinoma |
| LB63 colon carcinoma  |
| T cell leukemia       |

|   |
|---|
| - |
| - |
| - |
| - |
| - |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/02487

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93U, 93V; 435/7.23, 29, 240.2, 252.3, 320.1; 514/44; 530/350; 536/22.1, 23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS

search terms: tumor rejection antigen?, hla?, hlaa2, hla a2, tyrosinase, hlaa1, hla a1

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| Y         | SCIENCE, Volume 254, issued 13 Dec. 1991, van der Bruggen et al, "A Gene Encoding an Antigen Recognized by Cytolytic T Lymphocytes on a Human Melanoma", pages 1643-1647, see entire document.       | 1-20                  |
| Y         | NATURE, Volume 342, issued 23 November 1989, Bodmer et al., "Anti-HLA-A2 antibody-enhancement of peptide association with HLA-A2 as detected by cytotoxic T lymphocytes", pages 443-446, see p. 444. | 1-20                  |

|                          |   |                          |  |
|--------------------------|---|--------------------------|--|
| <input type="checkbox"/> | Further documents are listed in the continuation of Box C.  | <input type="checkbox"/> | See patent family annex.   |
| "                        | Special categories of cited documents:  | "I"                      | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| "A"                      | document defining the general state of the art which is not considered to be of particular relevance  | "X"                      | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| "E"                      | earlier document published on or after the international filing date  | "Y"                      | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L"                      | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "A"                      | document member of the same patent family  |
| "O"                      | document referring to an oral disclosure, use, exhibition or other means  |                          |  |
| "P"                      | document published prior to the international filing date but later than the priority date claimed  |                          |  |

|   |   |
|---|---|
| Date of the actual completion of the international search<br>13 JUNE 1994   | Date of mailing of the international search report<br>01 JUL 1994 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Hyosuk Kim<br>Telephone No. (703) 308-0196  |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/02487

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A01N 63/00, 43/04; A61K 37/00, 31/70; C07H 17/00, 19/00, 21/00; C07K 3/00, 13/00, 15/00, 17/00; C12N 1/20, 5/00, 15/00; C12Q 1/00, 1/02; G01N 33/53

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/93U, 93V; 435/7.23, 29, 240.2, 252.3, 320.1; 514/44; 530/350; 536/22.1, 23.1, 23.5